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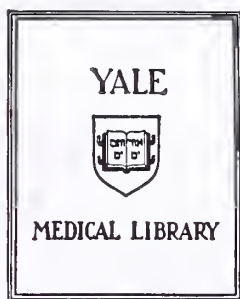
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STORAGE OF PLATELET CONCENTRATES
FOLLOWING ULTRAVIOLET-B IRRADIATION

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Yale University

1992




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STORAGE OF PLATELET CONCENTRATES
FOLLOWING ULTRAVIOLET-B IRRADIATION

A Thesis Submitted to the Yale University
School of Medicine in Partial Fulfillment
of the Requirements for the Degree of
Doctor of Medicine

Robert B. Johnson

1992

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ABSTRACT

Ultraviolet-B (UV-B) irradiation of platelet concentrates (PC's) may prevent the development of post-transfusion human leukocyte antigen (HLA) alloimmunization. This study evaluated the effect of UV-B radiation on stored PC's. Pooled PC's were irradiated at UV-B doses of 600, 2,400 or 10,000 mJ/cm² and stored up to 96 hours under standard blood bank conditions. Compared to non-irradiated room temperature and heat (37C) controls, the treated units showed no significant changes in platelet count, white cell count, discharge of lactate dehydrogenase, release of β -thromboglobulin, adenosine triphosphate, adenosine diphosphate, ammonia, glutamine, glutamate, hypoxanthine, pCO₂ or pO₂ at any time of storage following any of the three UV-B doses. However, after a dose of 10,000 mJ/cm², there were significant decreases in assays of platelet function, specifically osmotic recovery and morphology score. Metabolic systems were also affected, with a decline in pH and augmented glucose consumption and lactate production (p<0.05). These changes only appeared following 96 hours of post-irradiation storage, and were not seen in either control group. Thus, heat generated during irradiation did not affect in vitro platelet function. Using computer analysis of two-dimensional polyacrylamide gel electrophoresis patterns, PC's irradiated at 10,000 mJ/cm² and stored for 72 hours exhibited alterations in over 50 platelet proteins as compared to an age-matched non-irradiated control. It can be concluded that UV-B irradiation of PC's at doses up to 10,000 mJ/cm² does not induce significant metabolic, functional or protein derangements following short-term storage (24-48 hours). It is likely, however, that high dose UV-B irradiation followed by long-term storage (72-96 hours), will adversely affect some platelet metabolic and functional properties.

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INTRODUCTION

The availability of platelet concentrates (PC's) for routine transfusion has significantly reduced the morbidity and mortality due to hemorrhage in the thrombocytopenic patient. Perhaps nowhere has this treatment modality proven to be of greater value than in the support of patients with thrombocytopenia due to bone marrow failure (amgakaryocytic thrombocytopenia). For example, patients with malignant disease who receive myelosuppressive chemotherapy often need repeated platelet transfusions either to prevent or treat bleeding associated with periods of profound thrombocytopenia.¹ It is expected that such transfusion will result in an increase in the number of circulating platelets. The magnitude of this increase, known as the platelet increment, is of interest because it has been shown that there is a quantitative relationship between the number of circulating platelets, i.e. the platelet count, and the subsequent risk of clinical bleeding.² However, it is found in practice that not all PC transfusions result in the increment that is predicted based on the number of platelets transfused. This implies that the donor's platelets are rapidly removed from the recipient's circulation. If a patient receives several transfusions and consistently fails to achieve the expected increment following each infusion, then a state of refractoriness is said to exist.³

The causes of platelet refractoriness are most conveniently divided into those with immune and those with nonimmune mechanisms. The latter category includes patients with hypersplenism, fever, sepsis, hemorrhage, consumptive coagulopathies, e.g. disseminated intravascular coagulation, and hepatic veno-occlusive disease.¹ If all of these are excluded by history, physical examination and appropriate laboratory investigations, then an immune etiology should be considered. Several groups of platelet surface alloantigenic molecules, including those bearing ABO antigens and

platelet specific antigens have been implicated as causes of platelet refractoriness.^{4,5} However, it is generally believed that the majority of cases of immune refractoriness are the result of HLA sensitization, i.e. alloimmunization with resultant antibody production and proliferation of cytotoxic lymphocytes, to human leukocyte antigen (HLA) system Class I antigens, which are known to be present on platelets.⁴ Although there is no single test for the presence of such antibodies that offers 100% sensitivity and specificity,⁵ one study using a lymphocytotoxic (LCT) assay found that the presence of an LCT antibody was predictive of refractoriness to random donor PC transfusion in 88% of patients and the absence of an antibody presaged adequate increments in 77% of patients.⁶

The overall incidence of HLA alloimmunization in patients receiving multiple PC transfusions is unknown with estimates ranging from 30-60% depending upon the population studied.⁷ In addition, it is interesting to note that a clear dose-response relationship has not been found. In one study of patients undergoing remission induction chemotherapy for acute nonlymphocytic leukemia, the overall rate of alloimmunization with refractoriness was 38%. However, there was no correlation between the number of transfusions and the incidence or severity of alloimmunization.⁷ In a second study from the same group, it was found that of those patients who did not become HLA alloimmunized early in a long course of platelet transfusions, 92% never became refractory and those who became alloimmunized early tended to remain so.⁸ Another study of oncology patients did suggest that, in some cases, HLA antibody production may be transient and that HLA antibody may, in a small number of patients, even disappear despite continued random donor PC transfusion.⁹ There is, unfortunately, no reliable way of predicting which patients will become sensitized or which will spontaneously lose their sensitization. What is known is that the thrombocytopenic patient refractory to further transfusions presents a difficult management problem.²

One proven approach for treating HLA-alloimmunized patients involves the transfusion of HLA-matched PC's. The standard platelet transfusion consists of a pool of 6-10 individual random donor units of platelet concentrate (RDP's), each prepared from the blood of a different individual. It is possible, however, to prepare from a single individual a unit of PC, which contains a number of platelets equivalent to that contained in a 5-6 unit pool. This is known as a single donor platelet (SDP) unit and is obtained using automated or manual pheresis techniques (See Materials and Methods). This technology, combined with HLA typing, allows for the provision of an HLA-matched product that will hopefully be compatible with the patient's immune system by not presenting any antigens that will be recognized as foreign. Fortunately it has been found, in most cases, that matching of all donor and recipient HLA Class I loci is not required for successful transfusion.² Antigens at the HLA-C locus are not routinely typed because compatibility at this locus does not appear to be required to successfully transfuse an HLA alloimmunized recipient.² This leaves the HLA-A and -B loci to be matched and, while it may be difficult to find a truly compatible donor, the results of a complete HLA-A and -B matched transfusion are usually excellent. One series reported an 80% success rate with such transfusions in alloimmunized patients.¹⁰ When the match was less compatible, the rate of successful transfusion was appreciably lower, dropping to about 60% for the poorest matches.¹⁰

It is clear from these studies, however, that even when an ideal HLA-matched product is available, and this will not always be possible for those patients with rare HLA types, some refractory patients will still not benefit. While a number of these refractory patients have non-immune causes, it is believed that some of these cases of refractoriness are immune in origin, but may involve antigens other than the routinely typed HLA-A and -B loci.² For the alloimmune refractory patients unresponsive to the transfusion of HLA-matched PC's, and for those refractory patients

for whom a suitable HLA-matched donor cannot be found, there are no transfusion strategies available that reliably overcome immune platelet destruction,¹ nor is there any way of predictably reversing alloimmunization.¹¹ Although a low rate of spontaneous loss of immune refractoriness has been noted,^{8,9} prevention of alloimmunization clearly shows the most promise for maintaining responsiveness to platelet transfusions in chronically transfused patients.

One tactic seeks to prevent or delay the onset of alloimmunization by transfusing only SDP's. The rationale is that by minimizing the number of foreign antigens to which the recipient is exposed (as opposed to using RDP's), the time to develop alloimmunization will be delayed.³ Although, as noted above, a dose response relationship does not appear to govern the occurrence of alloimmunization when RDP's or SDP's are used,⁷ at least one prospective, randomized study has suggested that the use of SDP's may delay sensitization.¹² However, another randomized, prospective study showed no benefit of SDP's over pooled PC's.¹³ An extension of the SDP-only approach is the use of HLA-matched SDP's. Here, again, the concept is that by minimizing exposure to potentially immunizing antigens, HLA alloimmunization might be delayed or eliminated. A prospective, randomized evaluation of HLA-matched (degree of match not given) SDP's versus HLA-mismatched SDP's in oncology patients did show an advantage of the HLA-matched product in preventing alloimmunization.¹⁴ All five of the patients who developed persistent anti-HLA or other anti-platelet antibody during the study had initially been randomized to receive mismatched SDP's.¹⁴ However, three of these five patients continued to have adequate increments with mismatched SDP's and the study did not demonstrate a clear advantage in clinical outcome for the initial use of HLA-matched versus HLA-mismatched products. Given the overall low rate of alloimmunization (18%) in this study and the relatively small number of patients enrolled (78), it is difficult to draw conclusions regarding the potential of HLA-

matched SDP's to prevent or delay alloimmunization. In any case, the expense and effort required to provide HLA-matched SDP's for all patients likely to require multiple transfusions would overwhelm already overtaxed blood centers and hospitals. Only the largest facilities would have access to a donor base of several thousand individuals which would be needed to provide a varied pool of HLA-typed donors.³

A second approach attempts to reduce the host's immune response to the transfused mismatched antigens. Support for this hypothesis comes from the observation that leukemic patients tend to develop alloimmunization at a lower rate than do patients with aplastic anemia.³ It has also been noted that oncology patients receiving chemotherapy exhibit a lower than expected rate of Rh sensitization following Rh-incompatible platelet transfusions.¹⁵ If the difference is due even in part to the immune suppression associated with intensive cytotoxic therapy, then perhaps more selective modulation of host immunity might also be beneficial. An animal study using cyclosporine has demonstrated just such an effect. Of dogs treated with oral cyclosporine, 100% remained tolerant to repeated, unrelated donor, platelet transfusions.¹⁶ However, the potentially serious side effects of currently available immunosuppressive agents preclude their routine use for alloimmunization prophylaxis. Also, animal studies have shown that reversal of established alloimmunization via immune suppression is not as easily obtained as is prevention of aloimmunization.¹¹

A third approach seeks to reduce the immunogenicity of the transfused product. Studies have demonstrated that, although the antibodies that cause refractoriness are directed at HLA Class I antigens on platelets, the platelet itself is a weak immunogen.¹⁷ In fact, in animals transfused with platelet concentrates containing only platelets, alloimmunization to HLA antigens does not occur.¹⁷ This same study

demonstrated that only when white cells were present in the platelet transfusion did alloimmunization occur.¹⁷ Although the details of the process leading from PC transfusion to HLA Class I sensitization have yet to be fully elucidated, further investigation has revealed that a subset of leukocytes, those carrying HLA Class II antigens in addition to the Class I antigens present on all leukocytes and platelets, is responsible for the immunogenic nature of PC's.¹⁸

The white cells that carry Class II antigens are generically referred to as antigen-presenting cells (APC's) and, while macrophages, monocytes and B-lymphocytes have APC functions, it is believed that dendritic cells (DC's) are the primary cell type fulfilling this role in humans.¹⁸ As the standard blood bank methods of preparing PC's using differential centrifugation do not exclude white cells, significant numbers of so-called passenger leukocytes are present in the final product. This number is typically on the order of 10^8 - 10^9 per bag of PC prepared from a single unit of whole blood using standard centrifugation protocols, with less than 5% of these being DC's.¹⁸ In theory then, it should be possible to reduce or eliminate the immunogenicity of PC's if the passenger leukocyte APC's could in some way be removed or inactivated.

Physical removal of leukocytes may be accomplished by one, or a combination, of means: centrifugation, cell washing or filtration.^{19,20} However, these methods are not equally effective in removing leukocytes, sparing platelets and preventing alloimmunization. For example, a randomized, prospective trial of standard PC's versus PC's that had been leukocyte-depleted by centrifugation failed to demonstrate a lower rate of alloimmunization with the leukocyte-depleted product.²¹ Moreover, while centrifugation resulted in the removal of about 81% of the leukocytes, 27% of the platelets were also lost.²¹ In this study, each unit of PC contained approximately 10^7 leukocytes, i.e. depletion by less than 1 \log_{10}

as compared to a standard unit. Prevention of alloimmunization, it was hypothesized, may require that a larger fraction of the contaminating leukocytes be removed prior to transfusion. Indeed, studies using more elaborate centrifugation and washing protocols to remove larger numbers of white cells have shown a clear benefit of leukocyte-depletion in preventing alloimmunization.^{22,23} But, in all cases, the processing resulted in a 20-40% loss of platelets and was cumbersome. A method that is both more convenient and efficient would clearly be desirable.

Such a method exists in the form of so-called leukocyte depletion filters. Blood filtration technology now includes modification of cotton-wool and polyester filter surfaces with a variety of organic polymers that provide selective adsorption by cell type, e.g. leukocytes. These so-called third-generation filters offer selectivity and efficiency unavailable in the first- and second-generations.^{24,25} Third-generation filters reliably remove three logs of leukocytes (leaving about 10^6 leukocytes per PC unit) without any substantial decrement in platelet quantity or any detectable deterioration in quality.^{26,27} Several large patient trials have consistently shown a significant reduction, by up to 80%, in the frequency of alloimmunization when these filters are used.²⁸⁻³¹ These filters offer the convenience of bedside use and may become the standard of care for the patient beginning a long series of platelet transfusions who is not HLA alloimmunized at entry.¹⁹ It should be noted that leukocyte removal would not be expected to reduce the incidence of immune refractoriness due to non-HLA sensitization, e.g. platelet-specific antibodies, as APC's are not believed to be involved in non-HLA immunization. Clinical trials have verified this prediction.²⁸⁻³⁰

A final approach to reducing the immunogenicity of PC's is based on the observation that exposure to ultraviolet (UV) radiation abolishes the ability of lymphocytes to stimulate allogeneic cells in a mixed lymphocyte

reaction (MLR).³² As discussed above, it is believed that the presence of competent antigen-presenting cells (APC's) is essential for HLA immunization to occur.¹⁸ Studies using UV-A (320-400 nm) with a psoralen as a photosensitizing agent,³³ UV-B (280-320 nm)³⁴⁻³⁶ and UV-C (200-280 nm)¹⁸ have all confirmed that UV radiation impairs the ability of dendritic cells and other leukocytes to function as APC's. Further investigation has revealed that UV radiation has manifold effects and that it is likely that the impairment of APC function is due to a combination of factors. Among the reported adverse effects of UV radiation on APC's are: a direct reduction of cell viability via a cytocidal action (UV-B, UV-C);³⁷ an increase in intracellular calcium concentration in resting cells with a failure to mobilize additional calcium when stimulated (UV-B, UV-C);³⁸ a blockade of interleukin production, including IL-1, IL-2 and IL-6 (UV-A, UV-B, UV-C);^{33,39} a decrease in surface expression of intercellular adhesion molecule-1 (ICAM-1) (UV-B),^{40,41} which is thought to be important in the recognition of donor APC's by recipient T-lymphocytes.¹⁹ It has also been suggested that loss of HLA Class II antigens may occur following UV-B exposure.⁴² Other investigators, however, have not documented any decrement in Class II molecules.⁴³

Given the fact that UV exposure appears to abrogate the ability of APC's to present antigen to lymphocytes in culture, it was suspected that UV-irradiated PC's might also be less likely to cause alloimmunization. This has been confirmed in several animal studies. In both dog and mouse models, the use of UV-irradiated blood products is consistently associated with a lower rate of HLA alloimmunization for a given number of transfusions.^{14,33,44} A limited (32 patients) human trial, comparing the use of UV-B irradiated PC's to non-irradiated units, has demonstrated a 50% reduction in the frequency of HLA alloimmunization.⁴⁵ One of the animal studies also suggested that not only does UV radiation prevent HLA alloimmunization, but that the transfusion of blood products containing

UV-irradiated leukocytes may result in a broad state of tolerance in the recipient.¹⁶ As large human trials are currently underway to evaluate the efficacy of UV treatment for prevention of HLA-mediated refractoriness, the precise implications of an induced, non-specific immune tolerance of this type are unknown.¹⁹ Conceivably, both beneficial effects, such as enhanced rate of allograft acceptance, and adverse effects, such as greater susceptibility to infections, might be seen.

The foregoing discussion has focused on what UV radiation does to the passenger leukocytes. For UV irradiation to be a useful modality, however, it would have to be shown that the platelets themselves are not damaged in the process. Studies involving irradiation of human PC's have been reported using both UV-A and UV-B. In order for UV-A to efficiently abolish the APC activity of leukocytes, a psoralen must be added as a sensitizing agent.³³ One group has reported that there are no clinically significant adverse effects of such treatment on the in vitro function of platelets.⁴⁶ The parameters evaluated included pH, platelet count, lactate dehydrogenase leakage, morphology score, dense and alpha granule release and platelet aggregation studies (see Materials and Methods for explanation of these measurements). Because UV-B radiation is effective without the addition of a sensitizing agent, it has shown the most promise for clinical use. As there is neither standardized equipment nor accepted procedures, each UV-B irradiation system is different in some respects. Therefore, the doses of UV-B radiation reported should be taken to be approximations of the actual energy delivered to the leukocytes in the platelet concentrates. For example, a 2 mm thick plasma layer has been shown to allow only 0.03% transmittance of UV-B energy emitted by a common broad-band UV-B source.⁴⁷ Clearly, consistent results could only be expected for experiments conducted with a constant thickness of PC during irradiation. Other variables to be considered include type of plastic, UV-B source and use of agitation during irradiation. Also, it would be

important to consider whether the effects of UV-B irradiation are seen immediately and if storage intensifies or attenuates any damage that is caused.

One of the earliest studies reported used a UV-B dose of approximately 180 mJ/cm².³⁴ Although the platelet concentrate was irradiated in 15 mL aliquots, which is too small to be useful clinically, no adverse effects on platelet in vitro function were found immediately post-irradiation as determined by a panel of aggregation studies. The UV-B dose was noted to have effectively abolished the ability of treated lymphocytes to stimulate in an MLR. However, while in vitro aggregation is an important measure of platelet function, it is possible that other assays, or a period of storage following irradiation, might have revealed defects. Also, the small volumes of PC irradiated meant a very thin film of plasma was present in the bag during irradiation. This likely allowed a lower dose of UV-B to be used than would be required to treat clinical volumes of PC.

Pamphilon et al.⁴⁸ have reported irradiation of standard blood bag volumes of PC at 300 mJ/cm² followed by storage for up to 5 days -- the maximum currently approved storage period for PC's. This dose effectively blocked an MLR, but in vitro assays showed some potentially important differences in the irradiated versus non-irradiated units. Glucose consumption, lactate production, and alpha granule release were all greater statistically ($p < 0.05$) in the UV-B group and some of these changes became more prominent with increasing time of storage following treatment. No statistically ($p < 0.05$) significant changes in pH, osmotic recovery or aggregation were seen and one-dimensional polyacrylamide gel electrophoresis (PAGE) patterns were comparable. Also, in vivo autologous radiolabel studies gave similar results for recovery and survival. However, hemostatic efficacy was not assessed and the alterations in

glucose and lactate levels suggested that UV-B exposure may have induced changes in energy metabolism. To date, however, there are no published data regarding the specific effects of UV-B radiation on energy or intermediary platelet metabolism.

Other studies have used higher doses of UV-B energy. Andreu et al.⁴⁹ reported no adverse effects on in vitro aggregation for doses up to 3000 mJ/cm²; decreased aggregation responses were noted, however, for doses above that level. Autologous transfusion studies did consistently show a decreased time of survival for platelets treated with 1500 mJ/cm², but the number of subjects was too small to allow meaningful statistical analysis. Van Prooijen et al.,⁵⁰ at a dose of 6000 mJ/cm², found significant decrements in aggregability, a decrease in platelet thromboxane B2 content and increased macroscopic clumping. However, some of the techniques used were not consistent with clinical blood bank practice, e.g. irradiation in Petri dishes. Buchholz et al.⁵¹ used a UV-B dose of 2600 mJ/cm² and performed paired (same donor) transfusion studies comparing treated and untreated PC's. No differences were observed in in vivo survival, platelet count increment, or hemostatic efficacy as measured by correction of bleeding time. However, the increment and bleeding time studies did not involve storage following irradiation. The issue of post-irradiation storage is an important one. If UV treatment is proven in large clinical trials to be effective in reducing the rate of alloimmunization, then the ability to store an irradiated unit of PC would reduce problems with inventory control. This issue would arise when irradiated products go unused or if radiation treatment is performed at a few centers with distribution to outlying hospitals.

In summary, alloimmunization to HLA Class I antigens is an important cause of platelet transfusion refractoriness in multiply transfused patients. The mechanism of this phenomenon has yet to be fully

elucidated. Functional donor antigen presenting cells (APC's) have, however, been found to be an essential link in the immunization process. Based on this observation, two strategies have been proposed to reduce the immunizing potential of PC's. The first, leukocyte depletion by filtration, has already been shown in large clinical trials to reduce the frequency of HLA alloimmunization and to have no adverse effects on platelets. The second, UV-B radiation, which blocks APC function, is currently being evaluated in patient trials. Animal studies have suggested that UV abolition of APC function, as measured by an MLR, may be a method to reduce the incidence of alloimmunization. The experiments detailed below were, therefore, undertaken to evaluate what effects, if any, UV-B treatment and subsequent storage have on in vitro platelet function.

Previous published studies had evaluated some of the in vitro and in vivo effects of doses of UV-B radiation up to 6000 mJ/cm². However, the picture was incomplete in several important respects. Doses above 6000 mJ/cm² with subsequent storage had yet to be studied using UV irradiation procedures consistent with clinical transfusion practice, e.g. standard blood bag volumes. Also, while it is known that UV irradiation may lead to significant warming of the PC, this factor had not been systematically evaluated in any of the published studies.⁴⁹ In addition, there were several in vitro assays that had not been reported in the UV treatment literature. Platelet energy metabolism following UV-B treatment had been partially addressed by Pamphilon et al.⁴⁸ when glucose and lactate were measured. But, more detailed studies of energy metabolism, e.g. pCO₂, pO₂, adenosine diphosphate (ADP) and adenosine triphosphate (ATP), had yet to be done. Levels of key purine and ammonia metabolites had also not been reported for UV-treated PC's. Finally, while one-dimensional PAGE had been used to examine protein changes caused by a low dose (300 mJ/cm²) of UV-B radiation,⁴⁸ the more sensitive two-dimensional PAGE method had not

yet been applied. This thesis research was planned to examine these issues.

MATERIALS AND METHODS

Two sets of experiments were conducted. The first investigations, from June-August of 1989, involved only a single high dose (10,000 mJ/cm²) of UV-B radiation followed by a battery of in vitro assays. The second group of studies, conducted from November of 1991 to January of 1992, used three different doses (600, 2400 and 10,000 mJ/cm²) of UV-B to evaluate any dose-response phenomena and was likewise followed by a series of in vitro tests, including assays of intermediary metabolites. The cooperation and collaboration of several individuals were vital to the performance of many of these assays. Their specific contributions are detailed at the end of each section describing the materials and methods used in each set of experiments.

Part A: Storage of platelet concentrates after high-dose UV-B (10,000 mJ/cm²) irradiation

Standard units of platelet concentrate (PC's), collected from normal adult volunteer donors, were purchased from the American Red Cross Blood Services, Connecticut Region. These PC's were prepared by centrifugal fractionation of CPD (citrate-phosphate-dextrose)/AS-1 (adenine-saline) anticoagulated whole blood units according to standard Red Cross procedures.⁵²

For this study, 6 units of ABO-identical PC's (45-65 mL per unit), each in a 300 ml PL-732 blow-molded polyolefin bag (Baxter Healthcare Corporation, Fenwal Division; Deerfield, IL), were pooled aseptically into

a single 300 mL PL-732 bag using a plasma transfer set (#4C-2243, Baxter Healthcare). Under normal blood bank conditions, PC's may be stored for up to 5 days following collection. If the sterility of the container is violated, as by aseptic pooling, the shelf-life of the now open system is 4 hours (as mandated by FDA regulations). Therefore, for patient use, aseptic pooling would impose unacceptable time limitations. However, sterile connecting devices which allow 5 day storage of pooled PC's have become available since the first set of experiments; such a device was used in the second series of experiments (Part B). Sterile, 4 unit pools of ABO-identical PC's have been shown to be acceptable for clinical use following storage by both in vitro and in vivo evaluation, including the absence of any mixed lymphocyte reaction (MLR).⁵³ The PC's were pooled within 24 hours of collection, following completion of required viral antibody/antigen testing. After thorough gentle mixing, a sample was taken using a self-sealing sampling-site coupler (#4C-2405, Baxter Healthcare) and a plastic syringe with a 19-gauge needle; this was the pre-irradiation sample. Next, the 6 unit pool was split into a two unit volume to be stored without irradiation as a control and a 4 unit volume to be irradiated in a 500 ml PL-269 plastic bag (Baxter Healthcare). PL-269 is an experimental plastic which allows approximately 58% transmittance of the incident UV-B radiation.⁵¹ PL-269 is not, however, approved by the United States Food and Drug Administration for PC storage. The polyolefin plastic used for storage, PL-732, allows no more than 14% transmittance of UV-B radiation⁵⁴ and is, therefore, not suitable for irradiation. Therefore, PC's were irradiated in PL-269 and then transferred to PL-732 for storage. (See Figure 1 for flow diagram of PC handling)

The irradiation device used was an engineering prototype assembled by Baxter Healthcare for a clinical protocol (FRCP-0386) in which Yale-New Haven Hospital participated. It is a 12 bulb unit with 6 UV-B tubes

above a UV-transparent quartz glass plate and 6 tubes below. The bulbs (BLE-1T158, Spectronics Corp.; Westbury, NY), arranged in parallel, emit radiation in the wavelength band 280-320 nm. The radiation flux ($\text{mJ}/\text{cm}^2/\text{sec}$) across this band was measured using a radiometer with peak sensitivity at 310 nm (UVX Digital Radiometer, UVP Inc.; San Gabriel, CA). These data were then used to calculate the time required to deliver a total dose of $10,000 \text{ mJ}/\text{cm}^2$ to the surface of the plastic bag. This time was calculated to be approximately 17 minutes. The $10,000 \text{ mJ}/\text{cm}^2$ dose had been assigned to Yale-New Haven Hospital for patient trials by the sponsor (Baxter Healthcare). It should be noted that each irradiation system, i.e. plastic, UV radiation source, volume of plasma irradiated, agitation, is different, as neither equipment nor procedure has been standardized.

For all of the experiments, a 4 unit volume of PC was irradiated in a 500 mL PL-269 bag to maintain a consistent plasma thickness. Temperature fluctuations within the irradiation chamber were minimized by cooling fans. However, over the time required for irradiation, the temperature of the PC did increase to approximately 30°C . As PC's are normally stored at $20\text{-}24^\circ\text{C}$, this amount of warming was not felt to be harmful. It was not, however, controlled for in this series of experiments. The PC's were not agitated during irradiation.

After irradiation, the 4 unit test volume was split into two 2 unit volumes to be stored in standard 300 mL PL-732 bags with continuous gentle agitation provided by a 2 rpm circular rotator (Helmer Labs; St. Paul, MN). The control unit was also stored on the 2 rpm circular rotator. All PC's were kept at 22°C in a temperature controlled cabinet (Forma Scientific; Marietta, OH). The control and test PC's were sampled immediately post-irradiation (0-4 hours) and at 24 and 96 hours post-irradiation. The 96 hour time point corresponds to 5 days post-collection, i.e. the maximum allowed storage period.

The pH was measured at 25°C using a standard pH meter (PHM82, Radiometer; Copenhagen, Denmark). PC samples were diluted 1:10 with normal saline for platelet counting on a laser particle counter (ELT-800, Ortho Diagnostics Systems; Westwood, MA). White cell counts were run on undiluted samples on a laser particle counter (Coulter Counter T660, Coulter Electronics; Hialeah, FL). The dilution of PC samples for automated platelet counting was required to yield a count within the range where the machine provided reproducible results. White cell counts did not require dilution.

Morphology score was determined using the method of Kunicki et al.⁵⁵ This is a semi-quantitative assay of platelet integrity which consists of microscopic examination of unstained and unfixed samples of PC and assignment of a score based on counting 200 platelets with points awarded as follows: 4 for a disc, 2 for a sphere, 1 for a dendritic form and 0 for balloons or bizarre shapes. As a disc is the expected shape of a viable platelet, the highest possible score is, therefore, 800.

Osmotic recovery, also known as hypotonic shock response, is an assay that depends on the phenomenon of transient platelet swelling in a hypotonic medium. It was noted by Fantl⁵⁶ that placement of platelets in a hypotonic medium resulted in an immediate decrease in absorbance at 610 nm followed by a gradual increase in absorbance towards baseline. This has been interpreted to reflect initial platelet swelling due to free water influx followed by contraction with extrusion of water. The percent of osmotic recovery is defined as the fraction of initial absorbance attained in a given period of time following the addition of a hypotonic fluid. The procedure involves the measurement of baseline absorbance of a normal saline dilution of PC followed by measurement of absorbance at 10 minutes following identical dilution with distilled water. Absorbance was measured by a Varian DMS 90 Spectrophotometer (Varian Techtron Ltd.;

Mulgrave, Australia) at 610 nm with hardcopy tracing on a chart recorder (Linear Model 625, Varian Instrument Group; Sunnyvale, CA).

The tangent of osmotic recovery was also determined. This measures the tangent of the angle formed by the intersection of the horizontal baseline and a line tangent to the tracing of the increasing absorbance at the point of the tracing's steepest slope, i.e. the point where the absorbance is increasing most rapidly. A higher tangent, therefore, corresponds to a more rapid osmotic recovery by the PC. This is taken to be additional information regarding the total metabolic and contractile competence of the individual platelets present in the sample.

The percent discharge of platelet lactate dehydrogenase (LDH) was determined in two steps by measurement of LDH activity by an automated analyzer (Hitachi 717, Boehringer-Mannheim; Indianapolis, IN). First, the LDH activity is measured in a 1 mL sample of PC where the platelets have been lysed with Triton X-100 detergent (isooctyl phenoxypolyethoxyethanol, Sigma Chemical Company; St. Louis, MO). This result is taken to be the total LDH activity present in a 1 mL sample of that unit of PC. At each sampling time, the LDH activity present in 1 mL of platelet-poor supernatant plasma (prepared by centrifugation at 15,000 rpm for 3 minutes in an Eppendorf 5414 centrifuge, Eppendorf Gerätebau, GmbH; Hamburg, Germany) is measured. This result is taken to be the total extracellular LDH activity in 1 mL of that unit. Division of the extracellular supernatant level by the total LDH activity gives the percent of intracellular LDH released into the plasma at that time point. This has been shown to increase over time of storage and to increase when platelets are subjected to shear stress.⁵⁷ As LDH is present in the platelet cytosol and not in granules, LDH discharge has been used as an indicator of platelet lysis but not the release reaction.⁵⁷

The percent release of platelet β -thromboglobulin (β -TG) was likewise determined in two steps. This protein is known to be contained in platelet α -granules and to be released during platelet activation.⁵⁸ β -TG concentration was measured using a commercial ^{125}I radioimmunoassay kit (Amersham Corp.; Arlington Heights, IL). Release of β -TG has also been shown to occur during processing and storage.⁵⁷

Generation of complement fragments C3a and C5a was assayed using a commercial ^{125}I radioimmunoassay kit (Amersham Corp.). Some studies had suggested that UV-B radiation might quantitatively and/or qualitatively alter the expression of surface proteins (see Introduction). Such membrane effects could result in complement fixation with decreased in vivo survival either by opsonization of platelets or by the formation of transmembrane pores by the complement membrane attack complex (C5-C9).

Samples were prepared for 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE) as described in detail by Snyder et al.⁵⁹ Briefly, a 2.5 mL sample of PC is placed in a plastic tube containing buffer and chilled on ice to minimize platelet activation. Theophylline and EDTA are included in the buffer to stabilize membranes and reduce calcium fluxes, respectively. The sample is then centrifuged to remove erythrocytes. A buffer containing NaCl, glucose, adenosine, theophylline, phosphates, and two protease inhibitors, leupeptin and PMSF (phenyl-methylene sulfonyl fluoride), is added to the decanted platelet-rich plasma supernatant. The platelets are then sequentially washed and centrifuge-pelleted three times in ice cold buffer. While the final pellet contains mostly platelets, small numbers of erythrocytes ($< 1000/\mu\text{L}$) and leukocytes ($< 100/\mu\text{L}$) remain. The pellet is solubilized in 9M urea with NP-40, ampholytes (pH 9-11), 2-mercaptoethanol and leupeptin. Samples are then stored frozen at -30°C and warmed to room temperature before beginning the 2D-PAGE procedure.

The 2D-PAGE analysis involves a first dimension isoelectric focusing in 1.5 x 20 mm acrylamide tube gels followed by a second dimension separation by molecular weight on acrylamide gradient slab gels. The method is described in detail by Anderson and Anderson,^{60,61} as modified from O'Farrell.⁶² Briefly, a 10-15 μ L sample of solubilized platelets containing 30-40 μ g of protein is loaded onto the top of an acrylamide tube gel and subjected to electrophoretic focusing in a pH gradient resulting in separation of proteins by isoelectric point. The tube gel is removed from the focusing apparatus and laid across the top of a 1.5 mm thick 9-18% gradient acrylamide slab gel. The combined tube-slab gel is now subjected to electrophoresis in a sodium dodecylsulfate (SDS) buffer resulting in separation of proteins by molecular weight. Commercially available equipment (Electro-Nucleonics Inc.; Oak Ridge, TN) allows for up to 20 first dimension tube and 10 second dimension slab gels to be run simultaneously. This batch processing reduces gel to gel variability and allows meaningful comparisons to be made between gels of different samples run at the same time under identical conditions.⁵⁹ Following fixation in ethanol, acetic acid and sulfosalicylic acid, the slab gels are stained in batches with ammoniacal silver nitrate as described in detail in Guevera *et al.*⁶³

The 2D-PAGE system used, when combined with silver staining, has the power to display up to 10,000 individual protein spots and requires only about 1 ng of protein per spot to allow visualization.⁵⁹ Due to careful washing of the PC samples before electrophoresis, most of the major spots seen are platelet proteins and not contaminating plasma, leukocyte or erythrocyte proteins. To fully evaluate the complex gel patterns generated when whole solubilized platelets are electrophoresed, a computer image analysis system was used (Visage 2000, Bio Image; Ann Arbor, MI). This system allows detailed comparisons of spot number, density, size and position to be made between paired gels. The software has the flexibility

to allow for small differences in spot location between gels and, accounting for these discrepancies by digital mapping, to produce a spot by spot, quantitative comparison between the two gels. By scanning gels run on control and test samples from a single pool in the same 2D-PAGE and staining batches, a variety of comparisons were made, e.g. a non-irradiated control stored for 72 hours compared to its irradiated counterpart stored under identical conditions. Because the protein loading of gels was only approximately constant as judged by platelet counts and PC volumes, the full quantitative power of the computer system could not be applied. Therefore, the analysis of paired gels was confined to the qualitative accounting of spot numbers and relative intensity.

For each of the quantitative in vitro assays, the mean \pm 1 SD is reported. Statistical significance was taken to be $p < 0.05$ with paired t-tests used for comparisons.

All manipulations and assays were performed by this investigator with the following exceptions:

LDH by Clinical Chemistry at Yale-New Haven Hospital.

B-TG, C3a, C5a, and morphology score by Ms. Paula Napychank, Medical Technologist, at Yale-New Haven Hospital.

Computer gel scanning by Mr. Philippe Male of the Department of Cell Biology, Yale University School of Medicine.

Part B: Storage of platelet concentrates following variable dose UV-B (600, 2400 or 10,000 mJ/cm²) irradiation

Single donor platelet concentrates (SDP's) were prepared by apheresis collection from normal adult volunteer donors at Yale-New Haven Hospital (HIC Protocol #2000: Phlebotomy of Normal Volunteer Human Donors for Use in in vitro Studies) using a Fenwal CS-3000 Plus Blood Cell Separator with a separation chamber (TNX-6) and a collection chamber (A35)

(all by Baxter Healthcare Corp., Fenwal Division; Deerfield, IL). Informed consent, according to HIC protocol, was obtained prior to the procedure and all donors were screened for viral disease markers before donation. For the total of 10 donor PC units used to prepare 5 two unit pools, 7 men and 2 women participated with one of the men contributing one unit to each of two different pools.

The methods of automated blood processing available for the preparation of SDP's are discussed in detail by DePalma.⁵² Briefly, an apheresis device such as the Fenwal CS-3000 automatically performs the continuous flow centrifugal separation of a stream of citrate-dextrose anticoagulated whole blood into component parts, e.g. platelet-rich plasma, plasma, leukocytes and erythrocytes, and returns to the donor those fractions that will not be collected. By using a closed, sterile, non-pyrogenic collection system (Fenwal Closed System Apheresis Kit for Extended Platelet Storage and Plasma Collection, Baxter Healthcare), the PC could be stored for up to 5 days at 20-24°C with gentle agitation, i.e. the maximum currently approved duration of storage for PC's. Approximately $2-4 \times 10^{11}$ platelets were collected at each donation, as determined by sterile sampling, in a plasma volume of about 400 ml. This single donor PC was stored overnight as two 200 mL aliquots, each in a 1L PL-732 plastic bag (Baxter Healthcare) on a 70 cycle/minute linear platelet reciprocator (Melco Engineering Corp.; Glendale, CA) at 22°C in a temperature-controlled cabinet (Forma Scientific; Marietta, OH).

The day following collection, the two ABO-identical SDP's were pooled and gently mixed in a single 1L PL-269 bag (Baxter Healthcare). All transfers of PC between bags were carried out using a sterile connecting device (SCD 312; Haemonetics Corp.; Braintree, MA), which allows for sterile welds to be made in connection tubing thereby not compromising the shelf-life of a closed system.⁵³ For UV-B irradiation,

approximately 180 ml of PC were transferred to a 500 mL PL-269 bag. This corresponds roughly to the volumes irradiated in Part A. Three different doses (600, 2400 and 10,000 mJ/cm²) of UV-B radiation were used, administered by varying the time of exposure as judged by radiometer readings as described in Part A. The dose of 10,000 mJ/cm² required approximately 20 minutes, 2400 mJ/cm² approximately 5 minutes and 600 mJ/cm² about 1.5 minutes. The administration of the 10,000 mJ/cm² dose required slightly more time in Part B than in Part A due to a decrease in the intensity of the bulbs over a 30 month period. Following irradiation, the PC's were transferred back to standard 1L PL-732 bags (Baxter Healthcare) for storage on the linear shaker (Melco Engineering) at 22°C. (See Figure 2 for flow diagram of PC handling)

In addition to these three test units, two controls were used. For one of these, the heat control, a 130 mL volume of PC was placed in a 1L PL-269 bag and incubated at 37°C in a temperature-controlled room for a period of time corresponding to that required to complete the 10,000 mJ/cm² irradiation. As discussed in Part A, irradiation at a dose of 10,000 mJ/cm² results in warming of a 4 unit volume of PC to approximately 30°C. Incubation of the heat control in this experiment at 37°C for 20 minutes resulted in similar endpoint temperatures as determined by thermometry. The heat control was then split into two 65 ml volumes, each stored in a 300 mL PL-732 bag (Baxter Healthcare) at 22°C. The remaining 130 mL volume of PC was simply held in the 1L PL-269 bag used for pooling for 20 minutes at room temperature and then aliquoted for storage as two 65 mL volumes in 300 mL PL-732 bags. With these controls, the contribution of brief storage in PL-269 plastic and of warming to 30°C in PL-269 to any deleterious effects seen with the highest radiation dose can be examined.

The control and test units were sampled immediately post-irradiation (0-4 hours) and at 24 and 96 hours post-irradiation as described in Part

A. White cell count, morphology score, osmotic recovery, tangent of osmotic recovery, LDH discharge and β -TG release were determined as in Part A. Platelet counts were run as 1:10 dilutions of PC in normal saline on a laser particle counter (Coulter Counter T660, Coulter Electronics; Hialeah, FL). To evaluate the possibility of induction of metabolic derangements by UV-B irradiation, a number of metabolic parameters were examined -- pH, pO_2 , pCO_2 , glucose, lactate, ATP, ADP, ammonia, glutamine, glutamate, and hypoxanthine.

For determination of pH, pO_2 and pCO_2 , a sample of PC was injected at 22°C into a standard blood gas analyzer (Nova Stat, Nova Biomedical; Waltham, MA) as soon as was practicable after withdrawal from the storage bag. However, due to clinical priorities, the delay was sometimes up to two hours, during which the PC sample remained in a capped syringe at room temperature. Samples for lactate, glucose, hypoxanthine, ammonia, glutamine and glutamate were centrifuged at 3,000 rpm for 15 minutes in a Sorvall GLC-4 centrifuge with H-1000B rotor (Ivan Sorvall, Inc.; Norwalk, CT) to yield a platelet, erythrocyte and leukocyte poor plasma supernatant. This was then capped and frozen at -30°C for up to 8 weeks for later analysis. Lactate concentration was determined by an automated analyzer (TDX Analyzer, Abbott Diagnostics; Irving, TX), as was glucose (Astra-8, Beckman Instruments; Brea, CA). Hypoxanthine was measured by an enzymatic method relying on a change in absorbance at 280 nm as hypoxanthine is converted to uric acid by xanthine oxidase.⁶⁴ L-glutamine and L-glutamate were measured by an enzymatic method using glutaminase to convert L-glutamine to L-glutamate followed by oxidative deamination of L-glutamate to 2-oxoglutarate by glutamate dehydrogenase with the resultant increase in NADH concentration being monitored by absorbance change at 339 nm.⁶⁵ Ammonia was measured using a commercial enzyme kit based on the glutamate dehydrogenase reductive amination of 2-oxoglutarate with consumption of NADH monitored by absorbance change at 339 nm (Sigma

Chemical Company; St. Louis, MO). Samples for total ATP and ADP analysis were prepared by ethanol-EDTA solubilization of PC followed by centrifugation at 15,000 rpm for 1 minute in an Eppendorf 5414 centrifuge (Eppendorf Gerätebau, GmbH; Hamburg, Germany). The resultant supernatant was capped and frozen at -70°C for later analysis. ATP concentration was determined using a modified firefly luciferin/luciferase assay⁶⁶ with ADP concentration being calculated as the difference between ATP concentration as measured following addition of creatine phosphokinase and phosphocreatine to the system and that measured without such addition. Samples for hypoxanthine, L-glutamine, L-glutamate, ammonia, ADP and ATP were shipped overnight on dry ice to an outside lab (see below).

For each of the above in vitro assays, the mean \pm 1 SD is reported. Statistical significance as taken to be $p < 0.05$ with a repeated measures analysis of variance used for comparisons (SAS/STAT, Release 6.04, SAS Institute, Inc.; Cary, NC) run on an IBM PS/2 Model 80.

All manipulations and assays were performed by this investigator with the following exceptions:

Collection of PC's by Pheresis Service at Yale-New Haven Hospital.

LDH and glucose by Clinical Chemistry at Yale-New Haven Hospital.

β -TG and morphology score by Ms. Paula Napychank, Medical

Technologist at Yale-New Haven Hospital.

Blood gas analysis by Operating Room Staff at Y-NHH.

ATP, ADP, ammonia, hypoxanthine, glutamine and glutamate by Scott

Murphy, M.D. and his laboratory staff at the Cardeza Foundation

for Hematologic Research of Jefferson Medical College, Thomas

Jefferson University, Philadelphia, PA.

Statistical analysis by Ms. Karol Katz at the Biostatistics

Consulting Unit, Yale University School of Medicine.

RESULTS

Part A: Storage of platelet concentrates after high-dose ultraviolet-B irradiation

The numerical results of the in vitro assays performed in this series of experiments are seen in Table 1.

No statistically significant changes were seen in any of the parameters studied over the first 24 hours of storage following UV-B irradiation. In addition, no significant changes were noted in pH, platelet count, or white cell count over 96 hours of post-irradiation storage when the control (non-irradiated) and test (UV-B) groups were compared ($n=6$, $p>0.05$, NS). The control and test groups also did not exhibit differences at 96 hours in the discharge of LDH, release of β -TG or formation of complement fragments C3a and C5a ($n=6$, $p>0.05$, NS). By 96 hours of storage, however, the generation of C3a, LDH and β -TG had increased significantly for both groups ($p<0.05$). After 96 hours of storage, significantly lower morphology scores, osmotic recoveries and tangents of osmotic recovery were measured in the UV-B treated group as compared with the non-irradiated controls ($p<0.05$). Of the irradiated platelets evaluated at 96 hours, 50% showed a morphology score close to the lowest value (200) and an osmotic recovery of zero.

The 2-D PAGE pattern of paired irradiated and non-irradiated PC's stored for 72 hours is shown in Figure 3.

The computer program used to analyze the paired gels identified 745 discrete protein spots on each gel. The majority of these were essentially identical in location and intensity. The computer did find, however, 71 spots that differed when the test and control gels were

compared. Of these, 43 were present on the gel of the UV-B treated PC and were absent on the gel of the paired control PC; 19 spots were present on the control but not on the test gel; and 9 spots were found on both gels but differed markedly in intensity: 8 were darker on the test gel and 1 was darker on the control gel. The majority of the protein spots generated by the 2-D PAGE system have not been identified. It is, therefore, unclear whether the changes noted occurred in cytosolic, mitochondrial, granular, cytoskeletal or surface membrane proteins. Snyder et al.⁵⁹ provide an idealized computer-generated map of the protein pattern obtained when solubilized, whole human platelets are subjected to 2-D PAGE.

Part B: Storage of platelet concentrates after variable-dose ultraviolet-B irradiation

The numerical results of the in vitro assays performed in this series of experiments are seen in Tables 2 and 3.

The computer statistical software package used provided several analyses. The 96 hours of post-irradiation storage encompassed 3 sampling times (0-2, 24 and 96 hours post-irradiation). A repeated measures analysis of variance using univariate tests of hypotheses for within subject effects was used to determine whether the test (irradiated) and control (non-irradiated) groups differed in a given assay over all 3 sampling times. This is equivalent to plotting the mean values for a given assay at the 3 sampling times for the control and test groups on a linear scale and comparing the slopes of the resulting 2 curves. For comparisons between control and test groups in a given assay at each sampling time point, a repeated measures analysis of variance (pairwise comparisons) using a general linear models procedure with least squares means was employed. This is equivalent to plotting the mean values for a

given assay for the control and test groups on a linear scale and comparing the two groups only at each time point in isolation without reference to trends over time. The effect of time of storage was evaluated by a repeated measures analysis of variance using univariate tests of hypotheses for within subject effects.

The following comparisons between groups were made using the above procedures: room temperature control with heat control; room temperature control with each of the three UV-B doses (600, 2400 and 10,000 mJ/cm²); heat control with 10,000 mJ/cm²; 600 mJ/cm² with 2400 and 10,000 mJ/cm²; and 2400 with 10,000 mJ/cm². Reference will be made to differences that are seen between groups in comparisons over the full 96 hour storage period and in comparisons at the individual 0-2, 24 and 96 hour sampling points.

Over 96 hours of storage several assays did reveal statistically significant (n=5; p<0.05) differences between the irradiated and non-irradiated units. Importantly, when the non-irradiated room temperature and non-irradiated heat controls were compared, none of these differences was seen. This speaks strongly against a thermal effect of UV-B irradiation.

For pH, while all irradiated and non-irradiated groups showed a significant decline in pH over 96 hours of storage, a significant decrease in the 10,000 mJ/cm² group was seen when the room temperature control, heat control, 600 mJ/cm² and 2400 mJ/cm² were compared to the 10,000 mJ/cm² group. This effect was apparent by 24 hours of post-irradiation storage and marked by 96 hours of storage. No such decrement in pH was noted for the comparisons of room temperature control with 600 mJ/cm², room temperature control with 2400 mJ/cm² and 600 mJ/cm² with 2400 mJ/cm². This suggests that lower doses of UV-B radiation and 37°C incubation are

insufficient to cause a significant fall in pH, as the effect is observed only following the highest UV-B dose.

Measurements of pCO_2 , pO_2 , platelet count and white cell count revealed no significant differences between irradiated and non-irradiated units. Over 96 hours of storage, all groups showed statistically significant decreases in pCO_2 and platelet count; pO_2 was stable in all groups during storage.

Morphology scores were significantly lower in the 10,000 mJ/cm^2 group over 96 hours of storage only for the comparison of 2400 versus 10,000 mJ/cm^2 . In addition, the comparisons of room temperature control with 10,000 mJ/cm^2 and heat control with 10,000 mJ/cm^2 revealed significantly lower scores in the irradiated group at 96 hours of storage. This effect was not apparent at 24 hours of storage. All groups showed a drop in morphology score over 96 hours of storage.

The osmotic recovery assay failed to reveal any differences between the control (room temperature and heat) and irradiated units over the full period of storage. However, at 96 hours of storage, significantly lower osmotic recoveries were noted for the irradiated group in the comparison room temperature control versus 10,000 mJ/cm^2 .

Tangent of osmotic recovery, discharge of LDH and release of β -TG showed no significant differences between control (room temperature and heat) and irradiated units over 96 hours of storage or at any of the 3 time points of storage. All groups showed significant increases in generation of LDH and β -TG over 96 hours of storage.

Measurement of supernatant glucose concentration revealed a consistent effect of the 10,000 mJ/cm^2 dose over the 96 hour storage

period. Comparisons of the room temperature and heat controls with this high dose of UV-B and of the lower doses (600 and 2400 mJ/cm²) with 10,000 mJ/cm² all demonstrated significantly lower glucose levels in the 10,000 mJ/cm² irradiated units. This was not seen when the room temperature control was compared to either the 600 or the 2400 mJ/cm² dose. The decline in glucose concentration was most apparent at 96 hours post-irradiation. As all units started with equal platelet counts in identical suspending fluids and were stored and handled similarly, the fall in supernatant glucose concentration that was noted in the 10,000 mJ/cm² group can most likely be attributed to increased metabolism by the platelets present in those PC's. The possibility of gross bacterial contamination resulting in consumption of glucose can be ruled out as all PC units were examined microscopically (wet mounts) as part of the morphology scoring procedure. All groups showed a significant temporal decline in glucose concentration over the 96 hours of storage.

Measurement of supernatant lactate concentration yielded results consistent with those found with glucose. Comparisons of the room temperature and heat controls with the 10,000 mJ/cm² dose and of the lower doses (600 and 2400 mJ/cm²) with 10,000 mJ/cm² all demonstrated significantly higher lactate levels in the 10,000 mJ/cm² irradiated units over 96 hours of storage. In contrast to the results with glucose, however, this augmented lactate production also occurred in the 2400 mJ/cm² group when it was compared to the room temperature control and the 600 mJ/cm² group over 96 hours. Moreover, the rise in lactate levels in the 10,000 mJ/cm² group, as compared to the room temperature control or either of the 2 lower doses of UV-B, achieved significance by 24 hours of post-irradiation storage and persisted at 96 hours. Employing the same reasoning as applied to the glucose results, the higher supernatant levels of lactate can most likely be attributed to greater metabolic generation by the platelets in the 2400 and 10,000 mJ/cm² groups. All units showed

a significant increase in lactate levels over the 96 hours of storage.

Ammonia, glutamine, glutamate, ADP and ATP assays showed no significant differences between the control and irradiated groups over the full storage period or at any point of storage. All groups had significantly higher ammonia and glutamate concentrations after 96 hours of storage. Glutamine, ADP and ATP levels were noted to be significantly lower in all groups after 96 hours of storage.

Due to technical problems with the hypoxanthine assay in Dr. Murphy's laboratory, several data points were not obtained. Because of this, the data were not subjected to statistical analysis and firm conclusions cannot, therefore, be drawn from the available data. It would appear from inspection of the results, however, that a temporal effect of storage can be seen as all groups have markedly higher hypoxanthine levels at 96 hours than at 0-2 hours.

In summary, the investigations of Parts A and B of the study revealed that UV-B radiation has several potentially important effects on stored platelet concentrates. In Part A, significantly lower morphology scores, osmotic recoveries and tangents of osmotic recovery were noted after 96 hours of post-treatment storage in the irradiated ($10,000 \text{ mJ/cm}^2$) group of PC's as compared to the non-irradiated control group. Computer analysis of 2-D PAGE patterns revealed changes in over 70 protein spots after 72 hours of post-irradiation storage. Of these, 43 spots were present on the gels of irradiated PC's that were absent from gels of non-irradiated controls.

In Part B, the effects of a $10,000 \text{ mJ/cm}^2$ UV-B dose on osmotic recovery and morphology score were confirmed. Again, significant decrements in these 2 parameters were noted in the $10,000 \text{ mJ/cm}^2$ group at

96 hours of storage when compared to control groups. Assays for glucose and lactate revealed significantly augmented glucose metabolism in the 10,000 mJ/cm² group by 96 hours and significantly enhanced lactate generation in the 2400 and 10,000 mJ/cm² groups by 24 hours and continuing up to 96 hours. Commensurate with these metabolic changes, measurement of pH showed a significantly greater drop in 10,000 mJ/cm² group, as compared to the control groups, by 96 hours of post-treatment storage. Levels of other key platelet intermediary metabolites, including ammonia, glutamine, glutamate, ADP and ATP, did not indicate a specific effect of UV-B radiation.

DISCUSSION

The results of the assays performed in Parts A and B of this investigation provide a view of the effects of graded doses of UV-B radiation on the in vitro storage characteristics of PC's. While only a few of the parameters studied showed statistically significant changes in the irradiated units as compared to non-irradiated controls, the assays that did reveal changes may indicate potentially serious adverse effects of UV-B radiation on stored PC's. Importantly, the results of Part B appear to exclude heating during irradiation as a factor in inducing these alterations.

In Parts A and B, significantly lower morphology scores, osmotic recoveries and tangents of osmotic recovery (Part A only) were noted at 96 hours of storage following a 10,000 mJ/cm² dose of UV-B energy. One study has demonstrated a positive correlation between morphology score and in vivo survival of transfused platelets as measured by ⁵¹Cr labelling studies.⁵⁵ Scores below 400 were consistently associated with a significantly reduced post-transfusion circulating half-time. At 96 hours

post-irradiation, 50% of the treated units in Part A showed a morphology score close to 200, i.e. the lowest possible score.

The osmotic recovery phenomenon has been interpreted to reflect initial platelet swelling in the hypotonic medium due to free water influx followed by extrusion of this water with contraction by the platelets.⁵⁶ It has been demonstrated that the process of osmotic recovery requires both intact contractile proteins⁶⁷ and competent metabolic pathways.⁷³ Studies using inhibitors of energy metabolism, specifically glycolytic blocking agents, suggested that osmotic recovery is an energy dependent process.⁶⁷ In addition, the percent of osmotic recovery has been shown to correlate positively with in vivo survival as measured by ⁵¹Cr studies.⁶⁷ In Part A, fully 50% of the 10,000 mJ/cm² irradiated units had an osmotic recovery of zero at 96 hours and the mean osmotic recovery for the irradiated units at that time following treatment was only 17%. These PC's would be expected to have less than optimal survival characteristics following transfusion. The significantly lower tangents of osmotic recovery seen in the irradiated units at 96 hours in Part A are further evidence for the derangement of metabolic and/or contractile systems by UV-B radiation. However, as demonstrated in Part B, only the 10,000 mJ/cm² irradiated units appeared to suffer a significant decrement in osmotic recovery and then only after 96 hours of post-irradiation storage. The results of the measurements of key intermediary metabolites in Part B, specifically glucose and lactate, are consistent with the likelihood that a metabolic disturbance is responsible for the decreases in osmotic recovery seen with high doses of UV-B radiation.

A number of investigators have sought to elucidate the pathways of platelet intermediary metabolism.⁶⁸⁻⁷⁸ It has become clear that plasma glucose is an important substrate for energy metabolism, contributing approximately 15% of ATP requirements during normal aerobic storage

largely via glycolytic catabolism to lactate.^{70,72,74} Oxidative phosphorylation, perhaps utilizing plasma free fatty acids via β -oxidation,⁷⁷ contributes the remaining 85%.^{70-72,74} The importance of O_2 and CO_2 exchange across the storage plastic has been elegantly examined by Murphy and Gardner.⁶⁹ The existence of the Pasteur effect has also been demonstrated in stored platelets. This refers to the phenomenon of increased lactate production under anaerobic conditions as the platelet attempts to compensate for the loss of oxidative phosphorylation as an energy source by increasing glycolytic activity.^{69,70,72,74,75} The potential for high (>20 mmol/L) lactate concentrations to exceed plasma bicarbonate buffering capacity and, thereby, result in a damaging fall in pH has been documented.⁷⁰

The greater glucose consumption and lactate production seen in the irradiated units in Part B suggests that UV-B treatment, particularly the higher doses of 2400 and 10,000 mJ/cm², resulted in accelerated glycolytic metabolism. For example, the 10,000 mJ/cm² group consumed, by 96 hours an average of 5.7 mmol/L of glucose per unit and produced an average of 11.1 mmol/L of lactate per unit. (See Table 2) The ratio of lactate produced to glucose consumed is, therefore, approximately 1.95. This is close to the stoichiometry expected for glycolytic metabolism of glucose, i.e. 2 moles of lactate produced for each mole of glucose consumed. The room temperature control group consumed, by 96 hours, an average of 4.0 mmol/L of glucose per unit and produced an average of 6.6 mmol/L of lactate per unit (See Table 2). Here, the ratio of lactate produced to glucose consumed is 1.65. It would appear that in the control group, more glucose was consumed that can be accounted for by glycolytic production of lactate. This is not readily explained but may represent oxidative metabolism of glucose. In the 10,000 mJ/cm² group, almost all of the glucose consumed appeared as lactate. This reasoning holds if all lactate production is assumed to be a result of glycolysis.⁷⁴ Interestingly, the

ratios of lactate produced to glucose consumed for the heat control, 600 and 2400 mJ/cm² groups are 1.77, 1.72 and 1.86, respectively. The glucose and lactate values for the heat control and the 600 mJ/cm² radiation group were not statistically significantly different from the non-heated control values. Since the values for the 2400 mJ/cm² and the 10,000 mJ/cm² groups were different, this suggests a progressively greater shift to the glycolytic metabolism of glucose only with higher doses of UV-B radiation. Pamphilon et al.⁴⁸ also noted increased glucose consumption and lactate production in PC units irradiated at 300 mJ/cm² and subsequently stored for up to five days.

A significant decline in pH was also noted in the 10,000 mJ/cm² group in Part B. Studies have shown both decreased in vivo survival of transfused platelets and a fall in morphology score when pH falls below 6.4 or rises about 7.4 during storage.^{55,69} The more rapid decline in pH in the high-dose UV-B irradiated units can likely be attributed to the additional lactic acid produced by these PC's. However, the average pH at 96 hours in the 10,000 mJ/cm² group was 7.05, well within the range of acceptable. This suggests that the excess lactic acid did not exceed the available plasma bicarbonate buffering capacity. This is in agreement with the finding that only lactate concentrations above 20 mmol/L result in deleterious falls in pH.⁷⁰ The mean lactate concentration at 96 hours in the 10,000 mJ/cm² group was 15.0 mmol/L.

If, in fact, the changes in glucose metabolism can be attributed to an acceleration in glycolysis, the reason(s) for this acceleration must be sought. Broadly, potential explanations can be divided into two categories. First, the augmented glycolytic rate may be a reflection of additional energy requirements in irradiated platelets, i.e. the energy derived from the glycolytic pathway was required to supply greater than normal energy needs. In this case, glycolysis was recruited to provide

energy beyond that produced by oxidative phosphorylation. Second, the accelerated glycolytic rate in the irradiated PC's may indicate a failure of other energy sources, i.e. the energy derived from the glycolytic pathway is compensation for a failure of oxidative metabolism. Unfortunately, assays other than glucose and lactate that might have been expected to reveal effects of UV-B radiation on energy metabolism did not do so. This makes it difficult to distinguish between the two possibilities. It is possible, however, to suggest some plausible explanations.

Measurements of $p\text{CO}_2$ and $p\text{O}_2$, a by-product of and a substrate for, respectively, oxidative phosphorylation, failed to demonstrate a UV-B specific effect. As detailed in Murphy and Gardner,⁶⁹ the plasma $p\text{CO}_2$ and $p\text{O}_2$ reflect the aggregate oxidative metabolism of the PC, i.e. the inhibition of mitochondrial activity would be reflected as $p\text{CO}_2$ and $p\text{O}_2$ near atmospheric levels while accelerated oxidative phosphorylation would yield a higher $p\text{CO}_2$ and a lower $p\text{O}_2$. Neither of these phenomena was seen as an effect of UV-B irradiation as the results of $p\text{CO}_2$ and $p\text{O}_2$ were similar in both treated and control groups. However, the delay between sampling and measurement was occasionally up to 2 hours and this may have reduced the sensitivity of the assay for small differences in O_2 consumption and CO_2 production. Ideally, blood gas measurements should be made immediately (less than one minute) following sampling to minimize changes in $p\text{CO}_2$ and $p\text{O}_2$ caused by ongoing platelet metabolism in a closed system (syringes allow for little, if any, gas exchange).⁶⁹

Assays for total platelet ADP and ATP also failed to demonstrate any consistent effect of UV-B radiation. Both non-irradiated and irradiated groups had similar ADP and ATP content over the storage period. Also, assays for hypoxanthine and ammonia, products of adenosine catabolism, gave similar results for treated and untreated PC's. This again suggests

that overall platelet production and utilization of ATP was not significantly affected by UV-B exposure. An effect of UV-B radiation on energy metabolism cannot be excluded, however, as studies using the specific respiratory inhibitor antimycin A have shown that platelets can maintain a constant energy charge (total intracellular ATP) even in the face of a near complete blockade of oxidative phosphorylation.^{72,74} The source of ATP in this case is glycolysis and an acceleration of activity in this pathway is reflected as increased glucose uptake and lactate production.^{72,74} This is an example of the Pasteur effect of enhanced anaerobic metabolism in compensation for reduced aerobic metabolism.^{69,70} Recall that the irradiated PC's, particularly those exposed to higher doses of UV-B energy, showed a consistent and dose-related augmentation in glycolytic rate as shown by glucose and lactate levels. Based on these data, the rise in glycolytic activity in irradiated units may reflect inhibition of oxidative metabolism by UV-B radiation.

The other possibility, that increased glycolysis was required due to greater than normal energy requirements, is difficult to evaluate. The complex pathways of platelet activation, shape change and granule discharge are known to require energy.⁷³ But, to judge from release of β -thromboglobulin, a marker of platelet activation,⁵⁸ UV-B treatment did not induce significant activation. Other causes of increased energy requirements might include activation of proteolytic pathways, stimulation of mitochondrial DNA repair and transcription, and, generally, acceleration of any energy-dependent enzyme system. However, no assays relevant to these possibilities were evaluated in either Parts A or B. In addition, it is possible that the acceleration in glycolysis reflected both increased energy requirements and a failure of oxidative metabolism. As the total ADP and ATP concentrations were unaffected by UV-B treatment, no conclusions can be drawn about total energy needs with the evidence at hand. It also must be remembered that glucose, via glycolysis, supplies

only about 15% of the ATP needed by platelets during storage; the remaining 85% comes from oxidative metabolism utilizing free fatty acids.^{70-72,74} Definitive metabolic evaluation would require that assays of fatty acids and radiolabelling studies of substrate handling be included in the battery of in vitro assays.

UV-B radiation was shown in Part A, at a dose of 10,000 mJ/cm², to induce significant decreases in morphology score, osmotic recovery and tangent of osmotic recovery at 96 hours of storage following irradiation. These results were generally confirmed in Part B. Both morphology score and osmotic recovery have been shown to correlate positively with in vivo survival.^{55,67} A metabolic disturbance was suggested by the results of Part B. Specifically, an acceleration in glycolytic activity with maintenance of a constant ATP and ADP pool was found when irradiated and non-irradiated units were compared. A derangement in energy production or utilization is consistent with the finding of decreased osmotic recovery, a process known to be energy-dependent.⁷³ Recall that the increase in glycolytic activity may reflect compensation for a failure of oxidative metabolism or the recruitment of an additional pathway to supply greater than normal energy needs or a combination of both. Accordingly, the results of Parts A and B do not allow differentiation between these two possibilities.

As mentioned in the Introduction, the mechanism(s) by which UV-B radiation reduces the HLA immunizing potential of antigen-presenting cells has yet to be definitively identified. Alterations in protein expression and synthesis, e.g. ICAM-1 and interleukins, have received particular attention. The results of the 2-D PAGE studies in Part A suggest that platelet proteins themselves may be direct targets of UV-B radiation.

The 2-D gel pattern of a PC treated with UV-B radiation of dose of

10,000 mJ/cm² and stored for 72 hours showed changes in over 70 proteins as compared to an age-matched non-irradiated control. Of the 71 proteins with changes, 43 were present on the test gel that were not present on the control. It is possible to speculate that some of these 43 spots may represent proteolytic fragments induced by UV-B treatment. This could be an important effect of UV-B radiation as platelets do not have the ability to synthesize proteins in significant amounts. Indeed, platelet mitochondria are the only source of new platelet proteins.⁷⁹ Resolution of this question would require identification of at least some of the protein spots involved. This would necessitate isolation of these proteins in amounts sufficient to allow amino acid sequencing. At this time, it is unknown whether the proteins affected by UV-B treatment are membrane, cytosolic, mitochondrial, granular or cytoskeletal in origin.

The possibility that the expression of important surface membrane proteins is altered by UV-B irradiation was suggested by work done in a collaborating laboratory during Part A. (Teresa C. Wooten, 1992 Yale medical student thesis, "Storage of platelet concentrates after ultraviolet B irradiation: The effects on membrane glycoproteins GP IIb/IIIa and GP Ib.") These investigators found a significant decline in the platelet surface expression of GPIb, a glycoprotein essential to platelet adhesion, at 96 hours of storage following irradiation. Using flow cytometry and fluorescent monoclonal antibody labelling, a 60% reduction in surface GPIb was seen at 96 hours. Expression of a glycoprotein important in platelet aggregation, GP IIb/IIIa, was not affected by UV-B treatment and storage. The decrement in GP Ib expression could adversely affect in vivo function of the transfused platelets.

Previous studies of the effects of UV radiation on whole cells and organelles have suggested several mechanisms through which UV light can have deleterious effects. Two of these may be particularly relevant to

the issue of UV-B irradiation of PC's: mitochondrial damage⁷⁹⁻⁸³ and disruption of microtubules.⁸⁴ A study using far ultraviolet light, which includes UV-B, demonstrated rapid swelling and subsequent lysis of mitochondria in response to UV exposure.⁸⁰ It is currently unclear why lysis follows exposure but numerous explanations have been offered based on known actions of UV energy in other systems. These include induction of DNA-to-protein crosslinks, single strand DNA breaks, DNA intrastrand crosslinks and free radical generation with induction of proteolysis.^{79,81-83} If the enhanced rates of glycolysis noted in Part B occurred in compensation for an inhibition of oxidative phosphorylation, UV-induced mitochondrial damage is an attractive explanation. An important study to resolve this question might involve, in addition to more detailed metabolic assays, ultrastructural examination of platelet mitochondria with electron microscopy.

The potential importance of a disruption of microtubule function is reflected in the possible role of microtubules in the osmotic recovery phenomenon.⁶⁷ It is reasonable to assume that microtubules may be involved in other shape changing processes of platelets, e.g. activation. The decreases in osmotic recovery and morphology score seen following high-dose UV-B treatment and storage may reflect inhibition of microtubule function in addition to, or instead of, disturbances in energy metabolism. Only more elaborate studies of platelet microtubules could resolve this issue.

In summary, the observed effects of UV-B radiation on stored PC's, namely decreases in osmotic recovery, tangent, morphology score and pH with enhanced glycolytic activity and alterations in 2-D PAGE pattern may reflect one or more loci of action. Mitochondrial damage, disruption of microtubules, enhanced proteolysis and changes in surface membrane proteins are all possibilities. However, the results of this

investigation do not allow definitive conclusions to be made regarding these more general issues. The results do support the conclusion that UV-B irradiation of PC's at a dose of 10,000 mJ/cm² with subsequent 96 hour storage is likely to result in functional and metabolic derangements. Lower doses of UV-B radiation and/or storage for shorter periods is less likely to induce significant functional and metabolic changes.

Elucidation of the exact nature of the metabolic and functional changes found in this study will require detailed examination of platelet energy metabolism and identification of the proteins that are affected by UV-B radiation. The significance of the in vitro findings will also need to be evaluated by in vivo transfusion studies including survival, recovery and hemostatic efficacy assays. Preliminary in vivo studies involving UV-B treated PC's have been reported.^{45,48,51} The incidence of HLA alloimmunization does appear to be reduced when UV-B irradiated PC's are used and radiolabel survival, recovery and bleeding time data suggest adequate platelet function following transfusion. The present work points to the importance of further defining the mechanisms whereby UV-B radiation may damage platelets. Furthermore, there is a need to find the optimal UV-B dose that will reliably reduce the incidence of HLA alloimmunization and, simultaneously, allow treated PC's to be stored with minimal deterioration over their full shelf-life.

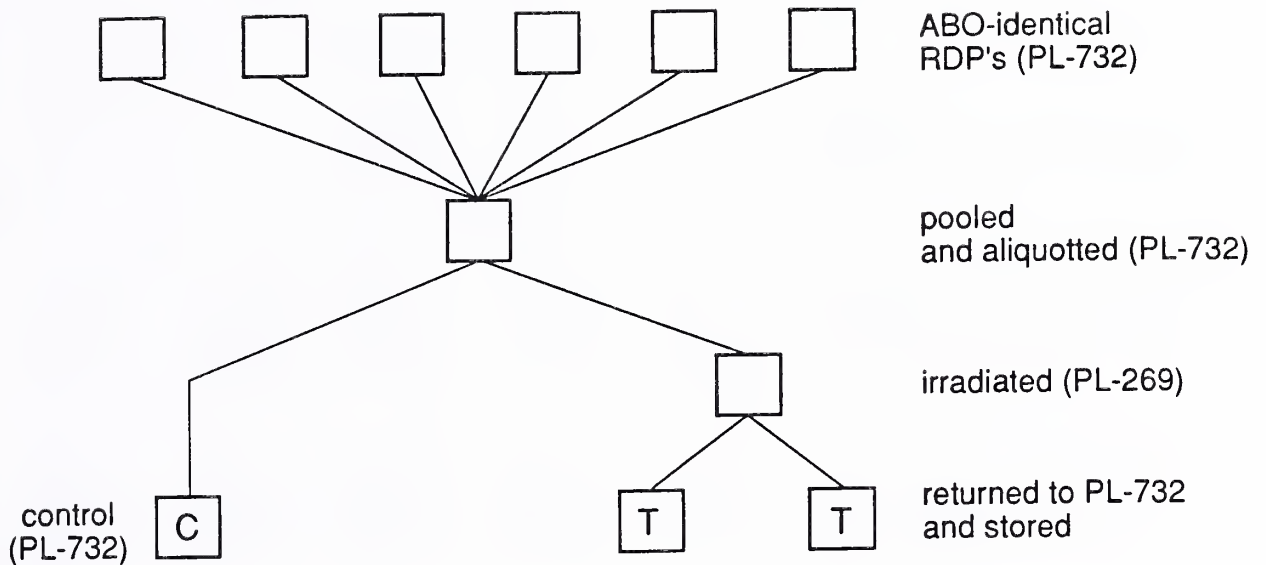


Figure 1: Flow diagram for handling of platelet concentrates in Part A. See text for explanation. "C" and "T" above correspond to control and irradiated test units, respectively.

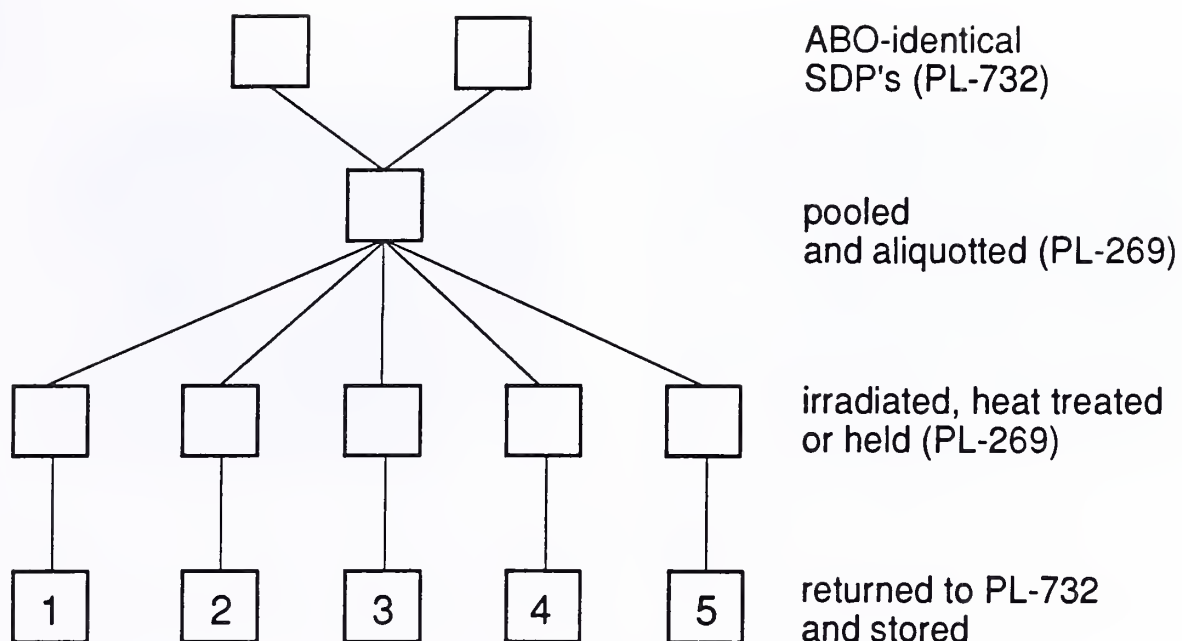


Figure 2: Flow diagram for handling of platelet concentrates in Part B. See text for explanation. Numbers above correspond to experimental conditions: 1: room temperature control; 2: 37°C control; 3: 600 mJ/cm² UV-B; 4: 2400 mJ/cm² UV-B; 5: 10,000 mJ/cm² UV-B.

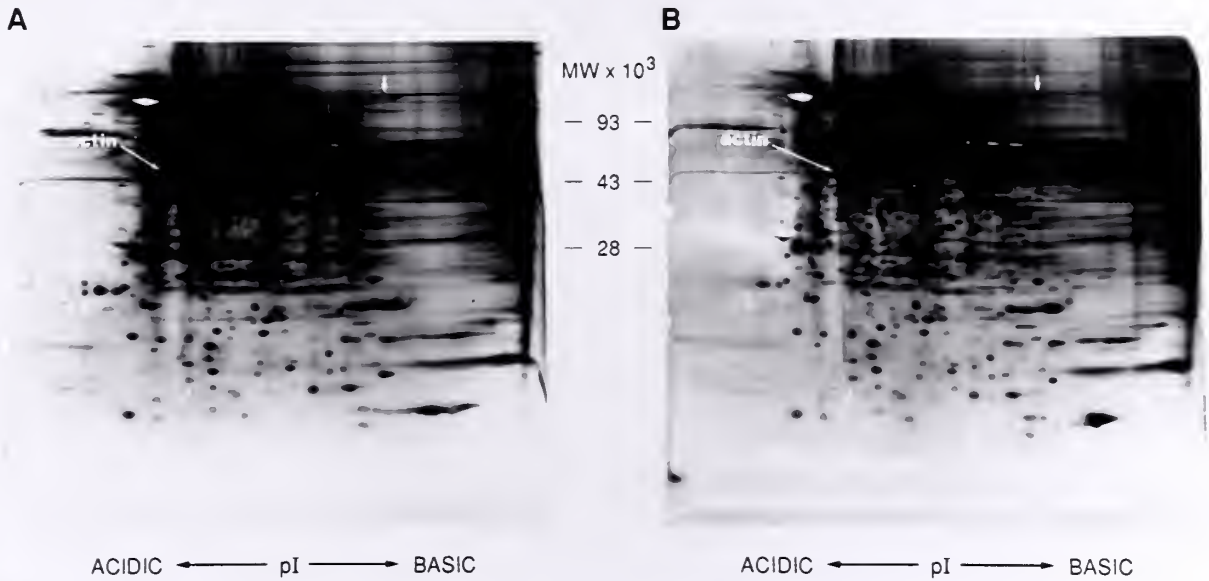


Figure 3: Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) pattern (silver stain) of paired samples of PC obtained from a single pool: A) control (non-irradiated) stored for 72 hours; B) irradiated at 10,000 mJ/cm² and stored for 72 hours. The isoelectric focusing axis (abscissa) places acidic proteins to the left and basic proteins to the right. The molecular weight axis (ordinate) places higher weight proteins at the top and lower weight proteins at the bottom. For reference, the molecular weight of actin is 43,000 Daltons. Although protein loading was only approximately equal, several easily visualized spots (see marked examples) appear to show a significant change in intensity.

Table 1: Storage of platelet concentrates after high-dose ultraviolet-B irradiation (Part A)

Assays*	SAMPLING TIMES							
	Before Irradiation	0-4 hrs post-irradiation		24 hrs. post-irradiation		96 hrs. post-irradiation		
		Control	Test	Control	Test	Control	Test	
pH (25°C)	7.33 ± 0.06	7.40 ± 0.08	7.38 ± 0.06	7.41 ± 0.06	7.31 ± 0.08	7.37 ± 0.11	7.22 ± 0.32	
Platelet count (x10 ³ /μL)	1590 ± 270	1510 ± 270	1390 ± 290	1500 ± 260	1430 ± 280	1360 ± 280	1360 ± 380	
White cell count(x10 ³ /μL)	2.0 ± 1.5	2.4 ± 1.0	2.0 ± 1.4	1.9 ± 1.4	1.7 ± 0.5	1.7 ± 0.5	1.7 ± 0.6	
LDH discharge (%)	6.2 ± 1.7	7.6 ± 1.5	7.0 ± 2.2	13.9 ± 10.7	9.2 ± 0.9	29.7 ± 13.0	42.6 ± 23.4	
β-TG release (%)	11.6 ± 4.3	14.0 ± 5.2	14.3 ± 5.4	20.6 ± 4.4	21.6 ± 5.2	29.4 ± 6.3	34.1 ± 10.9	
Morphology score**	646 ± 20	642 ± 31	583 ± 41	571 ± 41	553 ± 38	575 ± 66	253 ± 285	
Osmotic recovery (%)	78 ± 7	72 ± 6	63 ± 8	68 ± 14	67 ± 2	58 ± 22	17 ± 24	
Tangent of osmotic recovery	0.96 ± 0.07	1.0 ± 0.01	0.97 ± 0.07	0.91 ± 0.13	0.90 ± 0.12	0.73 ± 0.22	0.12 ± 0.17	
C3a (ng/mL)	832 ± 231	908 ± 393	1110 ± 798	1942 ± 762	2660 ± 979	9580 ± 3722	10,660 ± 4426	
C5a (ng/mL)	16.1 ± 9.6	18.3 ± 12.3	12.7 ± 6.7	16.5 ± 10.4	15.7 ± 9.7	19.4 ± 9.3	32.9 ± 18.5	

* = Results reported as mean ± 1SD; n=6

** = Max. 800

Table 3. Storage of platelet concentrates after variable-dose ultraviolet-B irradiation (Part B) - Platelet metabolic assays

Assays*	SAMPLING TIMES														
	0-2 hrs. post-irradiation					24 hrs. post-irradiation					96 hrs. post-irradiation				
	Control	Heat Control	600 mJ/cm ²	2400 mJ/cm ²	10,000 mJ/cm ²	Control	Heat Control	600 mJ/cm ²	2400 mJ/cm ²	10,000 mJ/cm ²	Control	Heat Control	600 mJ/cm ²	2400 mJ/cm ²	10,000 mJ/cm ²
Glucose (mmol/L)	17.1 ± 0.5	17.1 ± 0.6	17.0 ± 0.7	17.0 ± 0.7	16.9 ± 0.5	15.9 ± 0.4	16.0 ± 0.6	16.0 ± 0.6	15.9 ± 0.6	15.5 ± 0.7	13.1 ± 0.6	13.1 ± 0.6	13.1 ± 0.6	12.6 ± 0.7	11.2 ± 1.0
Lactate (mmol/L)	3.8 ± 0.2	3.7 ± 0.3	3.7 ± 0.2	3.7 ± 0.1	3.9 ± 0.2	5.7 ± 0.7	5.7 ± 0.4	5.2 ± 0.2	5.5 ± 0.2	6.6 ± 0.2	10.4 ± 0.8	10.8 ± 0.9	10.6 ± 0.6	11.9 ± 0.9	15.0 ± 1.0
Ammonia (μmol/L)	228 ± 106	193 ± 54	176 ± 32	194 ± 57	177 ± 46	324 ± 77	333 ± 69	274 ± 51	307 ± 98	310 ± 58	551 ± 114	597 ± 122	543 ± 92	542 ± 107	542 ± 77
Glutamine (μmol/L)	357 ± 44	337 ± 57	358 ± 56	336 ± 53	365 ± 48	257 ± 74	269 ± 57	297 ± 60	292 ± 55	299 ± 56	181 ± 76	178 ± 80	181 ± 68	177 ± 77	200 ± 96
Glutamate (μmol/L)	72 ± 37	69 ± 37	72 ± 34	93 ± 53	75 ± 31	151 ± 63	139 ± 40	108 ± 33	109 ± 36	116 ± 33	174 ± 52	190 ± 40	166 ± 25	166 ± 45	175 ± 44
ATP (μmol/10 ¹¹ platelets)	2.97 ± 0.25	3.43 ± 0.64	3.51 ± 0.65	3.33 ± 0.70	3.06 ± 0.50	2.70 ± 0.20	3.44 ± 0.61	3.43 ± 0.55	3.68 ± 0.98	3.39 ± 0.76	2.38 ± 0.50	2.77 ± 0.35	2.63 ± 0.29	2.75 ± 0.33	2.40 ± 0.57
ADP (μmol/10 ¹¹ platelets)	1.97 ± 0.59	2.22 ± 0.56	2.09 ± 0.78	2.31 ± 0.55	2.21 ± 0.56	1.63 ± 0.32	2.06 ± 0.62	2.11 ± 0.54	1.77 ± 0.56	2.00 ± 0.52	1.42 ± 0.47	1.58 ± 0.50	1.70 ± 0.45	1.80 ± 0.49	1.64 ± 0.41
Hypoxanthine** (μmol/L)	6.5 ± 3.5	7.7 ± 5.5	7.7 ± 4.8	9.2 ± 5.2	11.3 ± 4.6	16.2 ± 7.3	9.6 ± 4.4	8.7 ± 1.7	10.4 ± 2.1	12.6 ± 4.1	21.5 ± 4.5	26.3 ± 9.3	20.1 ± 2.2	23.0 ± 3.1	26.2 ± 5.6

* = Results reported as mean ± 1SD; n=5
** = n=3 to n=5 at various time points (see Results)

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